

Introduction

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1.1 Electromagnetic radiation

Light is a form of electromagnetic radiation, usually a mixture of waves having different wavelengths. Spectroscopic applications in structural laboratories are concerned with light from different wavelength intervals. Figure 1.1 presents an overview of different spectroscopic techniques and the energy intervals they operate in.

Many spectroscopic techniques in structural biology use light within the range of visible (Vis) colours extended on each side of the spectrum by the ultraviolet (UV) and the infrared (IR) regions (Table 1.1); these techniques are usually called spectrophotometric techniques.

1.1.1 Properties of electromagnetic radiation

The interaction of electromagnetic radiation with matter is a quantum phenomenon and dependent upon both the properties of the radiation and the appropriate structural parts of the samples involved. This is not surprising, as

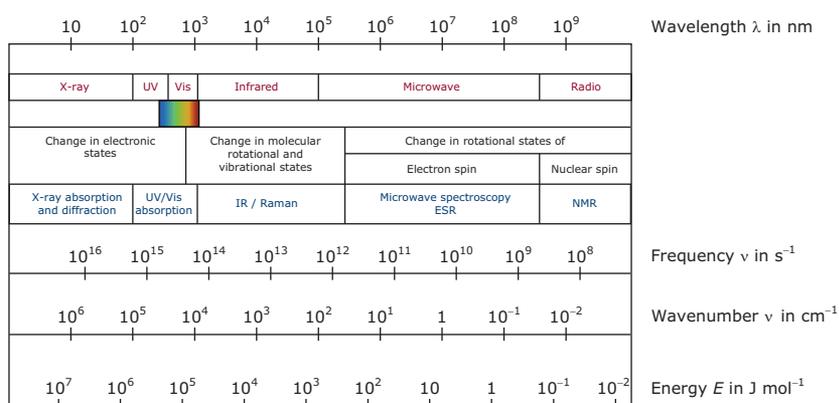


Fig. 1.1. The electromagnetic spectrum and its usage for spectroscopic methods.

Table 1.1. The three common light types for spectrophotometry.

	Wavelength (nm)	Wavenumber (cm ⁻¹)	Frequency (Hz)	Energy (eV)
UV	100–400	100 000–25 000	2.99×10^{15} – 7.50×10^{14}	12.4–3.1
Vis	400–700	25 000–14 286	7.50×10^{14} – 4.28×10^{14}	3.1–1.8
IR	700–15 000	14 286–667	4.28×10^{14} – 2.00×10^{13}	1.8–0.08

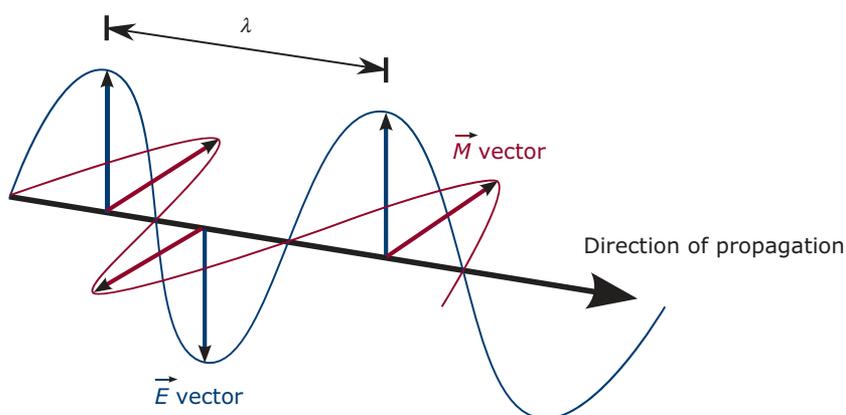


Fig. 1.2. Light is electromagnetic radiation and can be described as a wave propagating in space and time. The electric (\vec{E}) and magnetic (\vec{M}) field vectors are directed perpendicular to each other. For UV/Vis, circular dichroism and fluorescence spectroscopy, the electric field vector is of more importance. For electron paramagnetic and nuclear magnetic resonance, the emphasis is on the magnetic field vector.

the origin of electromagnetic radiation is due to energy changes within matter itself. The transitions that occur within matter are quantum phenomena, and the spectra that arise from such transitions are predictable in principle.

Electromagnetic radiation (Fig. 1.2) is composed of an electric vector (\vec{E}) and a perpendicular magnetic vector (\vec{M}), each oscillating in a plane at right angles to the direction of propagation. The wavelength λ is the spatial distance between two consecutive peaks (one cycle) in the sinusoidal waveform and is measured in multiples of nanometres (nm). The maximum length of the vector is called the amplitude. The frequency ν of the electromagnetic radiation is the number of oscillations made by the wave within the time frame of 1 s. It therefore has the unit of $1 \text{ s}^{-1} = 1 \text{ Hz}$. The frequency is related to the wavelength via the speed of light $c = 2.998 \times 10^8 \text{ m s}^{-1}$ (*in vacuo*) by $\nu = c\lambda^{-1}$. A related parameter in this context is the wavenumber

$$\tilde{\nu} = \frac{1}{\lambda}, \quad (1.1)$$

which describes the number of completed wave cycles per distance and is typically measured as cm^{-1} .

1.1.2 Interaction of light with matter

Figure 1.1 shows the spectrum of electromagnetic radiation organised by increasing wavelength, and thus decreasing energy, from left to right. Also annotated are the types of radiation, the various interactions with matter and the resulting spectroscopic applications, as well as the interdependent parameters of frequency and wavenumber.

Electromagnetic phenomena are explained in terms of quantum mechanics. The photon is the elementary particle responsible for electromagnetic phenomena. It carries the electromagnetic radiation and has properties of a wave, as well as of a particle, albeit having a mass of zero. As a particle, it interacts with matter by transferring its energy E :

$$E = \frac{hc}{\lambda} = h\nu, \quad (1.2)$$

where h is the Planck constant ($h = 6.63 \times 10^{-34}$ J s) and ν is the frequency of the radiation as introduced above.

When considering a diatomic molecule (see Fig. 1.3), rotational and vibrational levels possess discrete energies that only merge into a continuum at very high energy. Each electronic state of a molecule possesses its own set of rotational and vibrational levels. As the kind of schematics shown in Fig. 1.3 is rather complex, the Jablonski diagram is used instead, where electronic and vibrational states are schematically drawn as horizontal lines, and vertical lines depict possible transitions (see Figs 1.5 and 2.14).

In order for a transition to occur in the system, energy must be absorbed. The energy change ΔE needed for the transition is defined in quantum terms by the difference in absolute energies between the final and the starting state as $\Delta E = E_{\text{final}} - E_{\text{start}} = h\nu$.

Electrons in either atoms or molecules may be distributed between several energy levels but principally reside in the lowest levels (ground state). In order for an electron to be promoted to a higher level (excited state), energy must be put into the system. If this energy $E = h\nu$ is derived from electromagnetic radiation, this gives rise to an absorption spectrum, and an electron is transferred from the electronic ground state (S_0) into the first electronic excited state (S_1). Note that this requires an exact match of the photon energy with the energy difference between the two states that the transition is occurring between (resonance condition). The molecule will also be in an excited vibrational and rotational state. Subsequent relaxation of the molecule into the vibrational ground state of the first electronic excited state will occur. The electron can then revert back to the electronic ground state. For non-fluorescent molecules, this is accompanied by the emission of heat (ΔH).

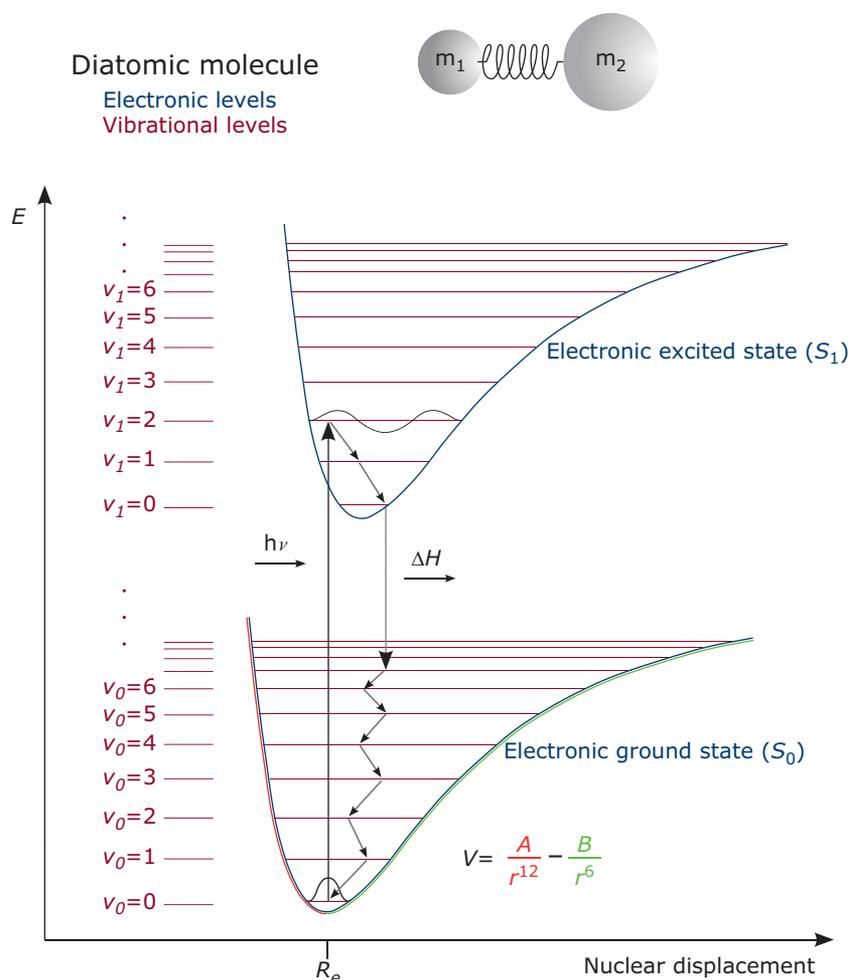


Fig. 1.3. Energy diagram for a diatomic molecule exhibiting rotation and vibration as well as an electronic structure. The energy of a system as a function of distance between two masses, m_1 and m_2 (nuclear displacement), is described as a Lennard–Jones potential curve (blue) with different equilibrium distances (R_e) for each electronic state. Energetically lower states always have lower equilibrium distances. The vibrational levels (quantum number ν ; red) are superimposed on the electronic levels. Rotational levels are superimposed on each vibrational level and not shown for reasons of clarity. The Lennard–Jones potential V is the most widely used model and consists of the van der Waals attractive term ($\sim r^{-6}$; green) and the Pauli repulsive term ($\sim r^{-12}$; red); A and B are constants.

Chromophores are the light-absorbing moieties within a molecule. Due to differences in electronegativity between individual atoms, they possess a spatial distribution of electric charge. This results in a dipole moment $\vec{\mu}_0$, such as for example the permanent dipole moment of the peptide bond

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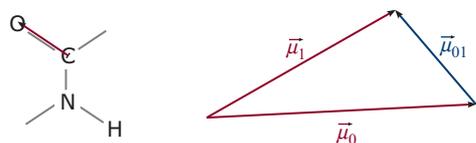


Fig. 1.4. Left: Dipole moment of the peptide bond. Right: The transition dipole moment $\vec{\mu}_{01}$ is the difference vector between the dipole moment of the chromophore in the ground state $\vec{\mu}_0$ and the excited state $\vec{\mu}_1$.

(Fig. 1.4). When light is absorbed by the chromophore, the distribution of electric charge is altered and the dipole moment changes accordingly ($\vec{\mu}_1$). The transition dipole moment $\vec{\mu}_{01}$ is the vector difference between the dipole moment of the chromophore in the ground and the excited state (Fig. 1.4). The transition dipole moment is a measure for transition probability. The dipole strength of the transition dipole moment, D_{01} , is defined as the squared length of the transition dipole moment vector:

$$D_{01} = |\vec{\mu}_{01}|^2 \quad (1.3)$$

Transitions with $D_{01} \rightarrow 0$ are called forbidden transitions and the probability of their occurrence is low. If $D_{01} \rightarrow 1$, the transition is said to be 'allowed' and occurs with high probability.

The plot of absorption probability against wavelength is called the absorption spectrum. In the simpler case of single atoms (as opposed to multi-atom molecules), electronic transitions lead to the occurrence of line spectra (see Section 2.1). Because of the existence of vibrational and rotational energy levels in the different electronic states, molecular spectra are usually observed as band spectra (for example Fig. 1.5), which are molecule specific due to the unique vibration states.

A commonly used classification of absorption transitions uses the spin states of electrons. Quantum mechanically, the electronic states of atoms and molecules are described by orbitals, which define the different states of electrons by two parameters: a geometrical function defining the space and a probability function. The combination of both functions describes the localisation of an electron.

In systems comprising more than one atom (molecules), the individual atomic orbitals combine into molecular orbitals (linear combination of atomic orbitals (LCAO); see Fig. 1.6).

Electrons in bonding orbitals are usually paired with anti-parallel spin orientation (Fig. 1.7). The total spin S is calculated as the sum of the individual electron spins. The multiplicity M is obtained by

$$M = 2S + 1. \quad (1.4)$$

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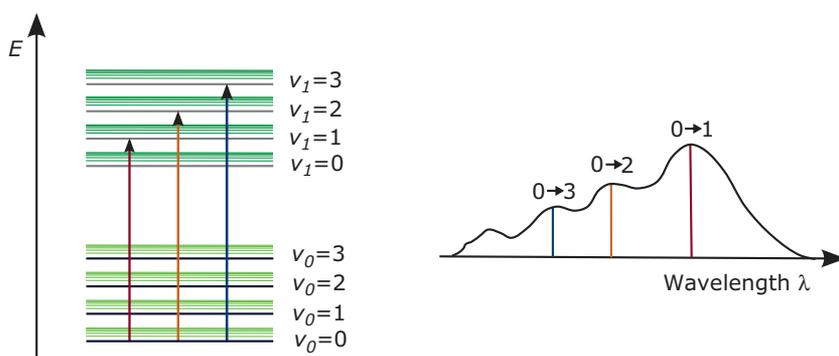


Fig. 1.5. Transitions in polyatomic molecules give rise to band spectra (as opposed to the line spectra observed with single atoms). Left: Vibrational (black, grey) and rotational (green) energy levels exist in the electronic ground state and excited state. Absorption of UV/Vis light leads to a general transition from the electronic ground to the excited state, but different vibrational and rotational levels are populated according to quantum mechanical rules. Right: As the lines for particular vibrational and rotational transitions are energetically close, they cannot be resolved individually and lead to the appearance of 'bands'. The main peaks represent the vibrational transitions. The rotational transitions are typically not resolved in UV/Vis absorption spectra.

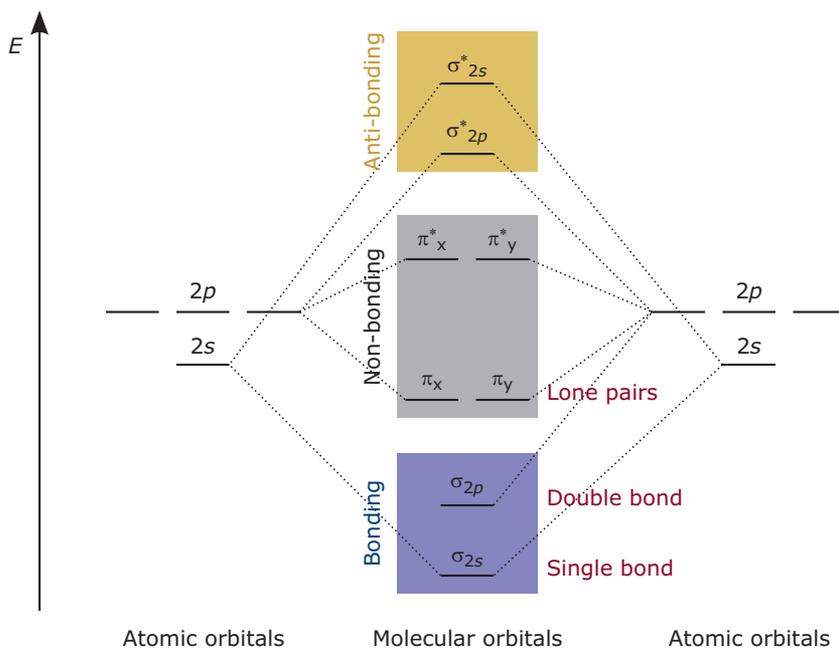


Fig. 1.6. Illustration of the linear combination of atomic orbitals (LCAO) to form molecular orbitals. Two atoms combine their $2s$ and $2p$ orbitals to form the molecular orbitals shown in the centre. The orbitals occupied by electrons responsible for single bond, double bond and lone electron pairs are indicated. Occupation of the anti-bonding orbitals leads to destabilisation of the bonds (bond cleavage).

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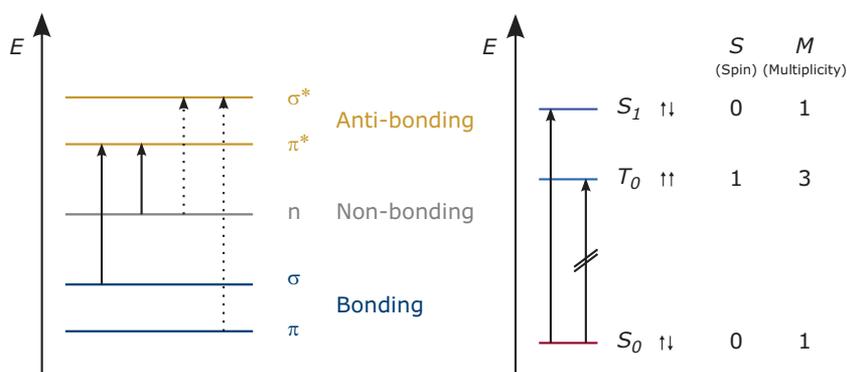


Fig. 1.7. Left: Energy scheme for molecular orbitals (not to scale). Arrows indicate possible electronic transitions. The length of the arrows indicates the energy required to be put into the system in order to enable the transition. Solid arrows depict transitions possible with energies from the UV/Vis spectrum for some biological molecules. The transitions shown by dotted arrows require higher energies (e.g. X-rays). Right: Due to quantum mechanical rules, only transitions with conserved total spin S are allowed. The transition between the two singlet states $S_0 \rightarrow S_1$ is therefore allowed; the transition from singlet ground state S_0 to the triplet state T_1 is forbidden.

For paired electrons in one orbital, this yields:

$$S = \text{spin}_{\text{electron1}} + \text{spin}_{\text{electron2}} = \left(+\frac{1}{2}\right) + \left(-\frac{1}{2}\right) = 0. \quad (1.5)$$

The multiplicity is thus:

$$M = 2 \times 0 + 1 = 1. \quad (1.6)$$

Such a state is thus called a singlet state and denoted 'S' (not to be confused with the total spin S above). Usually, the ground state of a molecule is a singlet state, S_0 .

In cases where the spins of both electrons are oriented in a parallel fashion, the resulting state is characterised by a total spin of $S = 1$, and a multiplicity of $M = 3$. Such a state is called a triplet state and usually exists only as one of the excited states of a molecule, e.g. T_1 .

According to quantum mechanical transition rules, the multiplicity M and the total spin S must not change during a transition. Thus, the $S_0 \rightarrow S_1$ transition is allowed and possesses a high transition probability. In contrast, the $S_0 \rightarrow T_1$ transition is not allowed and has a small transition probability. Note that the intensity of an absorption band is proportional to the transition probability.

Most biologically relevant molecules possess more than two atoms and therefore the energy diagrams become more complex than the ones shown

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in Fig. 1.3. Different orbitals combine to yield molecular orbitals that generally fall into one of five different classes (Fig. 1.7): s orbitals combine to the bonding σ and the anti-bonding σ^* orbitals. Some p orbitals combine to the bonding π and the anti-bonding π^* orbitals. Other p orbitals combine to form non-bonding n orbitals. The population of bonding orbitals strengthens a chemical bond, while the population of anti-bonding orbitals weakens a chemical bond.

1.2 Lasers

Laser is an acronym for *light amplification by stimulated emission of radiation*. A detailed explanation of the theory of lasers is beyond the scope of this textbook. A simplified description starts with the use of photons of a defined energy to excite an absorbing material. This results in elevation of an electron to a higher energy level. If, while the electron is in the excited state, another photon of precisely that energy arrives, then, instead of the electron being promoted to an even higher level, it can return to the original ground state. However, this transition is accompanied by the emission of two photons with the same wavelength and exactly in phase (coherent photons). Multiplication of this process will produce coherent light with an extremely narrow spectral bandwidth. In order to produce an ample supply of suitable photons, the absorbing material is surrounded by a rapidly flashing light of high intensity ('pumping').

Lasers are indispensable tools in many areas of science, including biochemistry and biophysics. Several modern spectroscopic techniques utilise laser light sources, due to their high intensity and accurately defined spectral properties. One of the probably most revolutionising applications in the life sciences, the use of lasers in DNA sequencing with fluorescent labels (see Section 2.3.6), enabled the breakthrough in whole-genome sequencing.

FURTHER READING

- Banwell, C. N. & McCash, E. M. (1994). *Fundamentals of Molecular Spectroscopy*, 4th edn. London: McGraw-Hill. (A readable account of spectroscopic principles.)
- Cantor, C. R. & Schimmel, P. R. (1980). *Biophysical Chemistry*, 1st edn. New York: Freeman. (A comprehensive reference in three parts.)
- Hoppe, W., Lohmann, W., Markl, H. & Ziegler, H. (1982). *Biophysik*, 2nd edn. Berlin, Heidelberg, New York: Springer Verlag. (A rich and authoritative compendium of the physical basics of the life sciences.)

Websites

Physics 2000: an interactive journey through modern physics, including topics such as waves, quantum mechanics, etc.: <http://www.colorado.edu/physics/2000/>

Applet: Spectrum: <http://lectureonline.cl.msu.edu/%7Emmp/applist/Spectrum/s.htm>

2

Spectroscopic methods

2.1 Atomic spectroscopy

Electronic transitions in single atoms yield clearly defined line spectra. In atomic emission spectroscopy (AES), these lines can be observed as light of a particular wavelength (colour). Conversely, black lines can be observed against a bright background in atomic absorption spectroscopy (AAS). The wavelengths emitted from excited atoms may be identified using a spectroscope with the human eye as the 'detector', or with a spectrophotometer. Both methods are used mainly for the analysis of metals, as well as some metalloids (such as boron, silicon, germanium, arsenic, antimony and tellurium) and a few non-metals (phosphorus).

Using light of high energy (e.g. X-rays), fluorescence from an electronic transition within individual atoms can be elicited.

2.1.1 Principles

In a spectrum of an element, the absorption or emission wavelengths are associated with transitions that require a minimum of energy change. In order for energy changes to be minimal, transitions tend to occur between orbitals close together in energy terms. For example, excitation of a sodium atom and its subsequent relaxation gives rise to emission of orange light ('D-line') due to the transition of an electron between the 3s and 3p orbitals (Fig. 2.1).

Electron transitions in an atom are limited by the availability of empty orbitals. Filling orbitals with electrons is subject to three major rules:

- Any one orbital can be occupied by a maximum of two electrons.
- The spins of electrons in one orbital need to be paired in an anti-parallel fashion (Pauli principle).
- Energetically equivalent orbitals are first to be filled with electrons of parallel spin; only then can a second electron with anti-parallel spin be added (Hund's rule).

Taken together, these limitations mean that emission and absorption lines are characteristic for an individual element.